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to 0.003, 0.004, and, finally at this time, is 0.009. I have successfully used corpuscles (1 per cent), which were kept 14 days at 1.6° C. suspended in isotonic salt solution. Lack of uniformity in results has caused me to discontinue using guinea-pig serum more than 24 hours old. No change has been detected in the strength of the specific antigen (alcoholic extract), which was extracted 10 months ago. The inactive patient's serum, if kept at refrigerator temperature and free from contamination, has given uniform results for lengths of time varying from 7 to 30 days.

CONCLUSIONS.

The complement content of fresh guinea-pig serum varies materially with different pigs.

More uniform and accurate results are obtained when the guinea-pig serum is standardized to known normal and syphilitic sera before doubtful samples are tested.

Hemolytic serum kept at uniform low temperature retains its activity for a relatively long time, altho it loses some of its original strength and needs restandardizing from time to time.

Suspensions of thoroughly washed red blood corpuscles (sheep) kept at 1.6° C. have been used up to 14 days after drawing with good results.

Samples of serum inactivated (56° C. one-half hour) and kept free from contamination remain unchanged for several days. This enables one to store samples and examine several at one operation.

Practical tests made with properly standardized reagents gave 95 per cent and 93⅓ per cent accurate results in known and doubtful cases respectively.

Negative reactions were obtained after vigorous specific treatment in 8 cases which gave positive reactions before treatment. A future publication will deal with this phase more extensively.

It would seem to be indicated by results from the limited number of cases tested that the complement fixation reaction, when carefully carried out and thoroughly controlled, is a reliable means for diagnosis in doubtful cases.

THE INFLUENCE OF AGE AND TEMPERATURE UPON THE POTENCY OF ANTIDIPHTHERIC SERUM AND ANTITOXIC GLOBULIN SOLUTION.*†

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INTRODUCTION.

FOLLOWING the work of Ehrlich, the determination of the potency of diphtheria antitoxin has been placed upon an exact and scientific basis. All the diphtheria antitoxin manufactured in the United States is now standardized in accordance with the unit furnished by the Hygienic Laboratory, which was actually promulgated April 5, 1905. Following the adoption of the American unit, antidiphtheric serum was admitted to the Eighth Decennial Revision of the United States *Pharmacopoeia.*

Considerable work has been done upon the duration of passive immunity following the administration of diphtheria antitoxin. It seems to be now generally accepted that passive immunity, following the use of diphtheria antitoxin, cannot be relied upon longer than three or four weeks.

While there has been a large amount of work done upon the above two phases of the manufacture and use of diphtheria antitoxin, there has been comparatively little done upon another most important part of the manufacture of this therapeutic product, i.e., an exact determination of the influence of age and temperature upon the potency of diphtheria antitoxin.

It has been the custom of most American producers of diphtheria antitoxin to date their serum to be returned or exchanged for fresh antitoxin in from 9 to 12 months after being placed upon the market. Lately some manufacturers have extended this period to 18 months or even two years, without, however, having any very exact data as to the keeping qualities of diphtheria antitoxin under adverse conditions.

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* Received for publication April 10, 1910.

† Read before the Laboratory Section of the American Public Health Association, Richmond, Va., October 20, 1909.

Consequently, there has been a demand on the one hand from the manufacturers of antitoxin for data on this subject for commercial reasons, and on the other hand from physicians from the standpoint of therapeutic efficiency. Some physicians even go so far as to doubt the propriety of using antitoxin, even in cases of emergency, when the return or exchange date on the package has been passed. I know of at least one instance in which a physician, in spite of the fact that he was not able to obtain fresh antitoxin, refused to use a package of serum on which the return date had expired.

Prior to 1906 there had been some work reported on the keeping qualities of diphtheria antitoxin, but most of it was open to the criticism either that the potency had not been determined in accordance with the Ehrlich method or that its strength had not been determined in the beginning or at the end of the experiment within sufficiently close limits to justify exact conclusions as to the influence of age and temperature. It would be manifestly unfair to draw conclusions as to the loss in strength of diphtheria antitoxin if a package of serum was placed upon the market containing an excess to allow for loss in potency, and when returned from the market and tested for its decrease in potency, not to take into account this excess. For example: If a package of serum labeled "to contain 1,000 units," but actually containing 1,200 units, or 20 per cent excess to allow for loss in strength, was placed upon the market and then returned at the end of a year and tested to ascertain whether it still contained 1,000 units, the package might be found to still contain 1,000 units of serum in spite of the fact that it had lost the 200 units excess placed in the package.

Early in the fall of 1906 the question of the deterioration of diphtheria antitoxin incident to age and temperature was taken up in the Hygienic Laboratory and all the licensed manufacturers in the United States were invited to contribute serum for this purpose, and several of them did so. In all, there were 18 different lots of serum furnished, 14 being untreated serum and 4 serum concentrated by the Gibson process. It is to be regretted that the number of both kinds of serum was not larger, but it is believed that the number was sufficient to provide data as to the influence of age and temperature upon the potency of antitoxin.

PREVIOUS WORK.

Alba[1] gives the results of retests of ten lots of diphtheria antitoxin and concludes that antitoxin retains its potency unchanged for a long time. His results are of little value to us, as we know that the method of testing the potency of serums, prior to the use of the present method of Ehrlich, was not accurate.

Marx[2] gives the results of the retesting of 1,104 samples of serum at the Institut in Frankfurt sent there between March, 1895, and June, 1903. Of this number, only 34, or 3.08 per cent, were withdrawn on account of a decrease in potency. The losses by months were as follows: in 3 to 4 months, 2 samples; 6 to 10 months, 9 samples; 10 to 20 months, 23 samples. He concludes that the loss in potency, when it does take place, is gradual and that the lack of confidence in old sera is not well founded.

An editorial in the *Journal of the Royal Army Medical Corps*[3] gives the results of the retesting of six lots of serum that had been for a year either in military hospitals at home or had made one or two voyages to India on transports during that time. Only four of the six lots had lost in potency, the loss varying from 5 to 18 per cent. The conclusion drawn was that antitoxin retains its activity little, if any, impaired at least 12 months.

Miller[4] tested the strength of a large number of packages of serum returned to the manufacturers. The time between tests varied from seven months to more than six years. Of 82 lots tested, 30 per cent showed a loss in potency varying from 0 to 50 per cent. It was found that the high potency serum underwent a greater decrease in potency than the low; but that every package, when retested, contained at least the number of units claimed on the label due to the excess allowed by the manufacturer for deterioration.

Layson[5] studied the decrease in potency of diphtheria antitoxin kept under known conditions from one to four years. His first tests were made on 14 lots of serum kept in bulk for one year at 4° C., then kept in a cool room (temperature not stated) for a year, after which they were kept at room temperature for the remainder of the time. The loss on retesting from three years eight months to one year three months later was from 40 to 12.5 per cent.

The second series of tests were on 16 lots of serum in sealed bulbs kept in the ice-chest at 4° C. from three years seven months to two years six months. The maximum loss was 16.66 per cent, half of the number showing no loss.

Seven lots that had been on the market and were returned after the expiration of the time limit were tested and found to have lost from 16.7 per cent to no loss in two lots. Of seven lots kept in bulk in the ice-chest from 10 months to 16 months, only one showed loss.

Kinyoun and Hitchens[6] tested the loss in strength of 100 lots of serum that had been returned after being on the market and kept under unknown conditions from 13 to 27 months. They state that in the original tests the unit strength was determined within 50 units, but that on the retest within 10 units. It was found that the loss in unit value ranged from 0 to 48.6 per cent, and that at least 65 per cent of the packages, irrespective of the unit strength, showed a depreciation in value of 25 per cent for each 12 to 15 months. They conclude that an excess of 24 per cent should be added to all serum on the market to allow for deterioration in potency. This study is of particular value on account of it having been made on a large number of samples kept under commercial conditions.

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[1] *Centralbl. f. Bakt.*, Orig., 1898, 23, p. 934.

[2] *Festschrift von Robert Koch*, 1903, pp. 451–62.

[3] "Diphtheria Antitoxin" (editorial), *Jour. Roy. Army Med. Corps*, 1904, 2, pp. 601–2.

[4] *Centralbl. f. Bakt.*, Orig., 1905, 38, pp. 233–36.

[5] *Amer. Med.*, 1905, 10, pp. 746–48.

[6] *Centralbl. f. Bakt.*, Ref., 1907, 40.

METHOD.

The serum was furnished by the contributors either in small vials containing about 3 c.c. each or in the usual "ready-to-use" syringe in which antitoxin is commonly marketed in the United States. In this way an unopened package was used for each successive test.

As soon as possible after the serum was received at the laboratory its potency was determined within 10 units. The entire lot of serum was then divided into three portions: One portion was kept at room temperature, one in the cold room at 15° C., and the third in the ice-chest at 5° C., all three portions, of course, being kept in the dark.

Every six months after the beginning of the experiment the potency of each portion was again determined within 10 units. All tests subsequent to the first were so made that the highest potency for which it was tested was the strength determined at the previous test. In this way there was always shown in each test the decrease in potency since the previous one and an approximate indication was had of the probable decrease for the ensuing six months. In every instance two guinea-pigs were placed on each dose, in order that any irregularities might be indicated and avoided. All the guinea-pigs used were of standard weight, between 250 and 280 gm., and were from the stock raised at the laboratory and known to have no inherited immunity to diphtheria toxin. At least two and usually three controls were given the L+ or test dose of toxin used against one standard unit, and if an irregularity determined by the death of the controls was noted the test was repeated.

An effort was always made to place the lower limit of tests so that some of the animals that failed to die an acute death would develop paralysis, in order that data might be obtained as to the ratio between the least amount of antitoxin conferring complete protection and that permitting the development of paralysis.

On account of the large number of guinea-pigs required for this work, some 4,000 altogether, serious inroads were made upon our stock of guinea-pigs; but it was felt that the importance of the work fully justified their use.

All dilutions and inoculations were made by me individually, so that any personal factors that might have entered into the work were largely eliminated.

The following charts show the results of the tests of 18 lots of serum in semi-annual periods when kept at 5° C., 15° C., and room temperature.

The serum kept at room temperature was in the large bacteriological laboratory room. In the winter the temperature of this room was about 70° F.; in the summer it varied with the diurnal weather conditions of Washington, D.C., sometimes reaching a maximum temperature of 95° to 100° F.

The legend at the top of each chart shows the age of the serum when the experiment was begun, potency at that time, and the kind and amount of preservative used.

The three curves on each chart show the percentage of loss at each successive observation from the original strength, and the actual strength at the last test.

Charts 1 to 14 give the results obtained in the testing of untreated antitoxic serum; Charts 15 to 18, results with serum precipitated and concentrated by the Gibson process.

THE KEEPING QUALITIES OF DRIED ANTITOXIN.

It has been shown by others as well as by me that diphtheria antitoxin in the liquid serum inevitably loses in potency by age, but there does not seem to have been any work reported as to the keeping qualities of antitoxin in the dried state. In May, 1904, I prepared a large amount of dried diphtheria antitoxin to be used in the preparation of the American standard diphtheria unit. Most of this serum was kept in the special bulb that I designed for this purpose,[1] under vacuum and P_2O_5. A few bulbs were kept without the acid and not under vacuum. The dried serum at the time of its preparation in 1904 contained 5,250 units per gm. Recently I tested the potency of some of the serum kept since 1904 at 5° C., but not under vacuum and P_2O_5, and found it still to contain 5,250 units per gm.; in other words, the dried serum in 5½ years is of the same potency as when first prepared. It is to be regretted that some of the same serum was not placed at room temperature and at 15° C. in order to determine if it retains its potency as well under these conditions as at 5° C. I am inclined to believe that such would have been found to be the case, or at most, the loss would have been very slight.

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[1] M. J. Rosenau, "The Immunity Unit for Standardizing Diphtheria Antitoxin," *Bull. 21, Hygienic Laboratory*, February, 1905.

As the dried serum apparently retains its potency with but slight impairment, it would seem that dried diphtheria antitoxin is peculiarly suited for shipment to the tropics, or for use on ships that make long voyages. The only drawback to its use appears to be in the making of a sterile solution, and this could largely be overcome by aseptic precautions by the user and the producer of the serum.

SUMMARY AND DISCUSSION.

A study of the preceding charts shows that the average loss in percentage strength of the 14 untreated sera for three years at room temperature was 44.2 per cent; at 15° C., 24.4 per cent; at 5° C., 16.7 per cent.

The average percentage loss in potency of the same sera for two years at room temperature was 32.8 per cent; at 15° C., 18.5 per cent; at 5° C., 12.4 per cent.

The average percentage loss for one year at room temperature was 18.7 per cent; at 15° C., 10.2 per cent; at 5° C., 6.7 per cent.

The average loss in percentage strength of the 4 sera concentrated by the Gibson method for two years at room temperature was 25.8 per cent; at 15° C., 18.8 per cent; at 5° C., 12.9 per cent.

The average percentage loss of the same sera for one year at room temperature was 16.4 per cent; at 15° C., 10.5 per cent; at 5° C., 8.8 per cent.

From the above it will be seen that there was but little difference in the keeping qualities of the untreated serum and that concentrated by the Gibson process except for the serum kept at room temperature for two years, in which case there was a difference in favor of the Gibson serum of about 8 per cent. It would seem that the yearly loss in potency is about 20 per cent, altho occasionally it may go as high

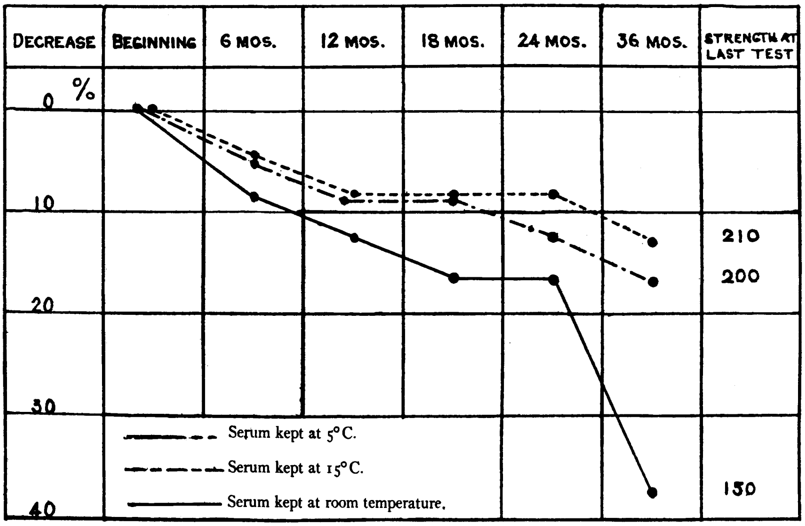

CHART 1.—Serum No. 1,110, F.S. Blood drawn August 29, 1904. Serum preserved with 0.4 per cent trikresol. 549 days from collection of serum to beginning of experiment. Strength when experiment was begun, 240 units per c.c.

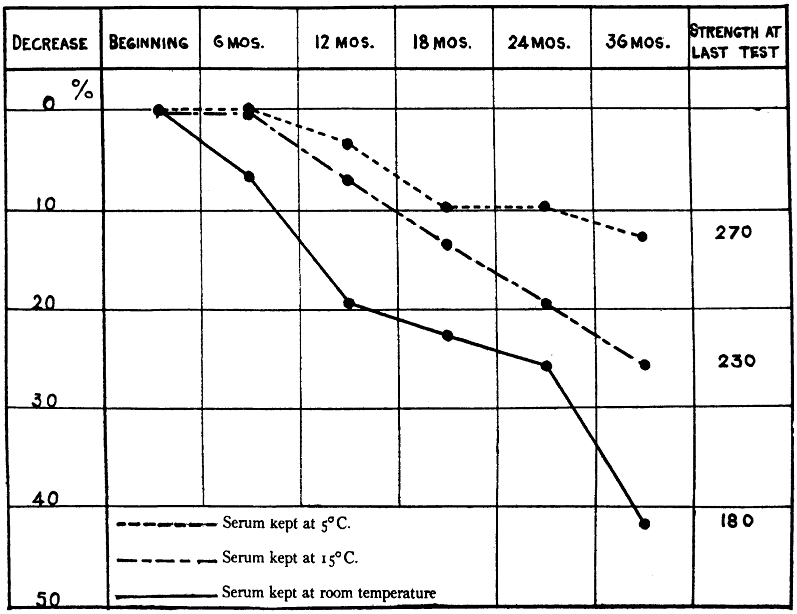

CHART 2.—Serum No. 08034, P.D. Blood drawn September 16, 1905. Serum preserved with 0.4 per cent trikresol. 70 days from collection of serum to beginning of experiment. Strength when experiment was begun, 310 units per c.c.

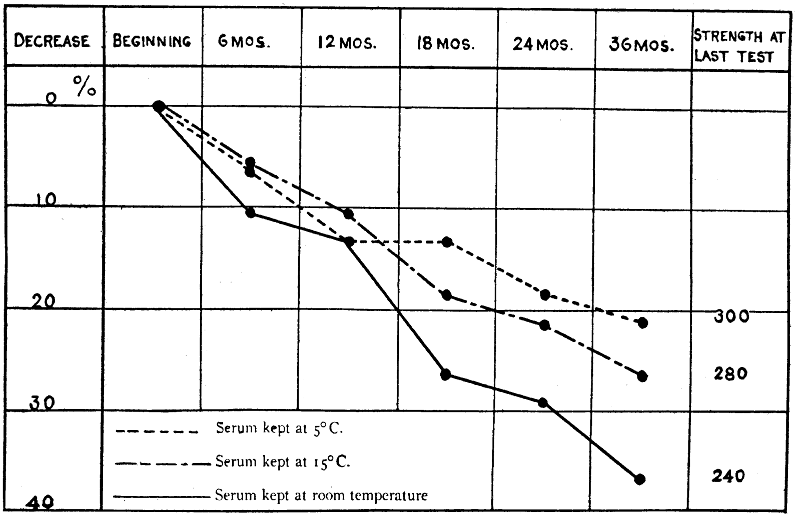

CHART 3.—Serum No. 192, H.M.A. Blood drawn August 14, 1905. Serum preserved with 0.4 per cent trikresol. 191 days from collection of serum to beginning of experiment. Strength when experiment was begun, 380 units per c.c.

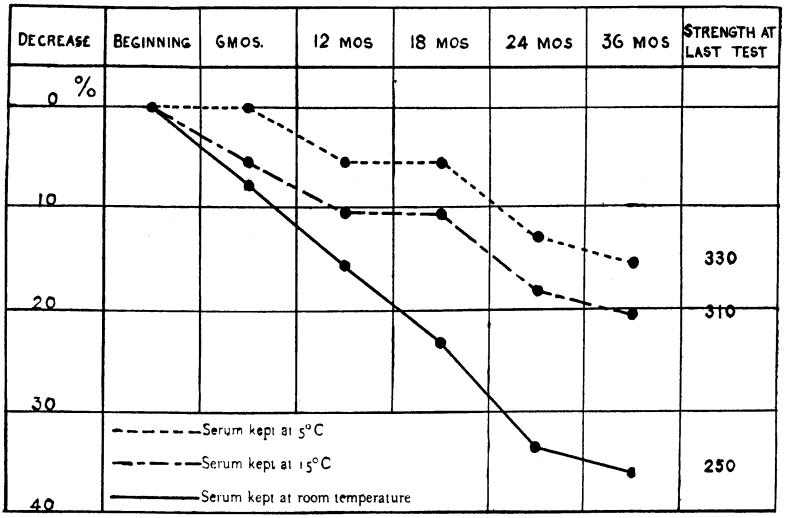

CHART 4.—Serum No. 08033, P.D. Blood drawn September 16, 1905. Serum preserved with 0.4 per cent trikresol. 80 days from collection of serum to beginning of experiment. Strength when experiment was begun, 390 units per c.c.

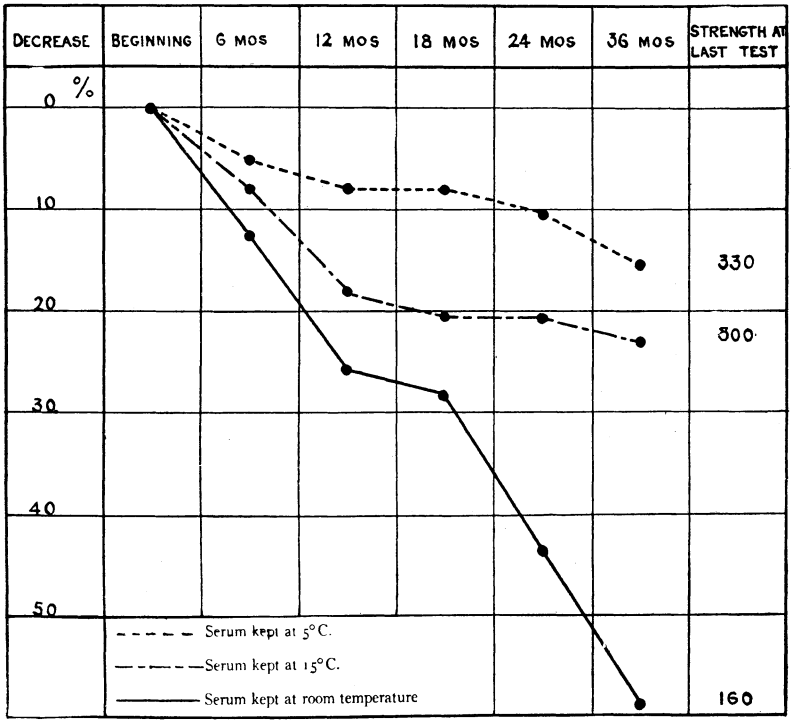

CHART 5.—Serum No. 08023, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 151 days from collection of serum to beginning of experiment. Strength when experiment was begun, 390 units per c.c.

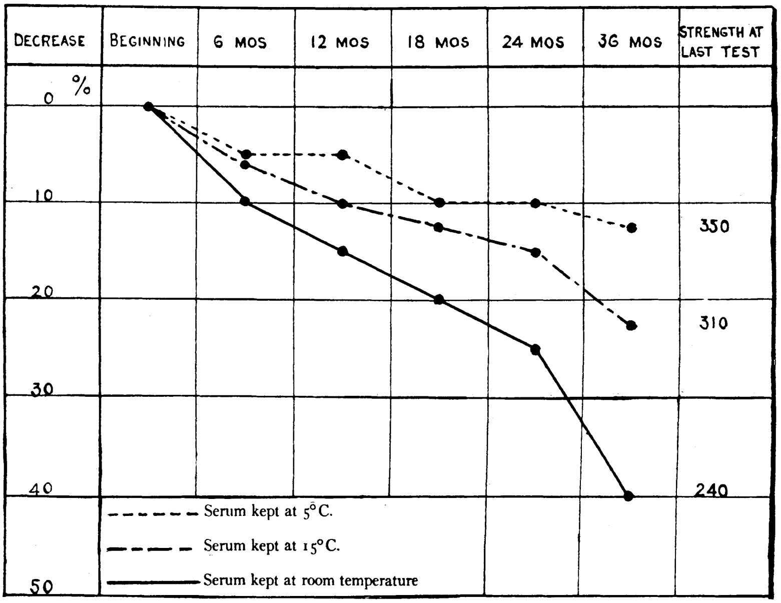

CHART 6.—Serum No. 1,351, F.S. Blood drawn February 13, 1905. Serum preserved with 0.4 per cent trikresol. 385 days from collection of serum to beginning of experiment. Strength when experiment was begun, 400 units per c.c.

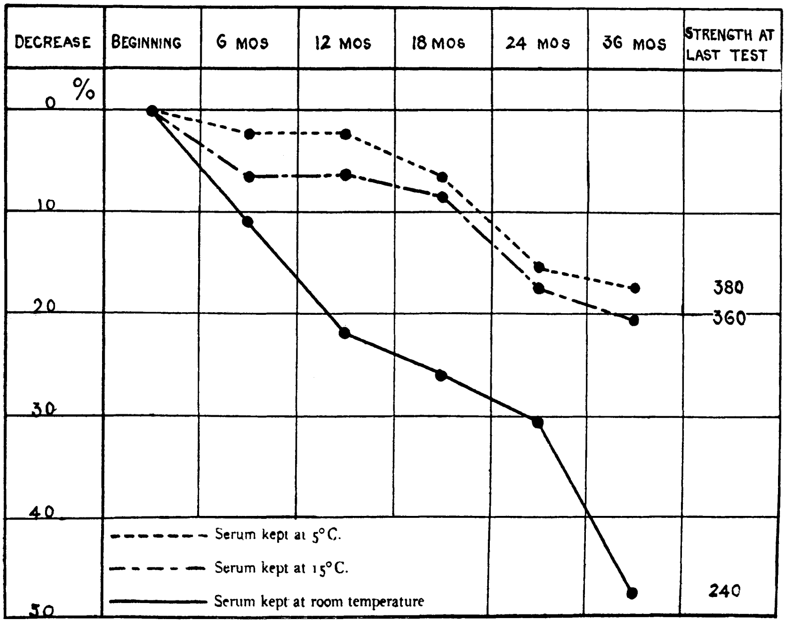

Chart 7.—Serum No. 08021, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 101 days from collection of serum to beginning of experiment. Strength when experiment was begun, 460 units per c.c.

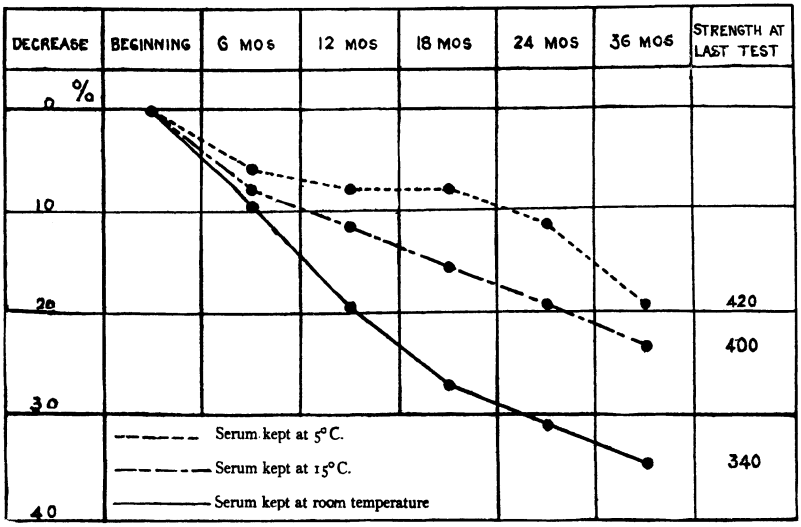

Chart 8.—Serum No. 1,429, F.S. Blood drawn May 8, 1905. Serum preserved with 0.4 per cent trikresol. 253 days from collection of serum to beginning of experiment. Strength when experiment was begun, 520 units per c.c.

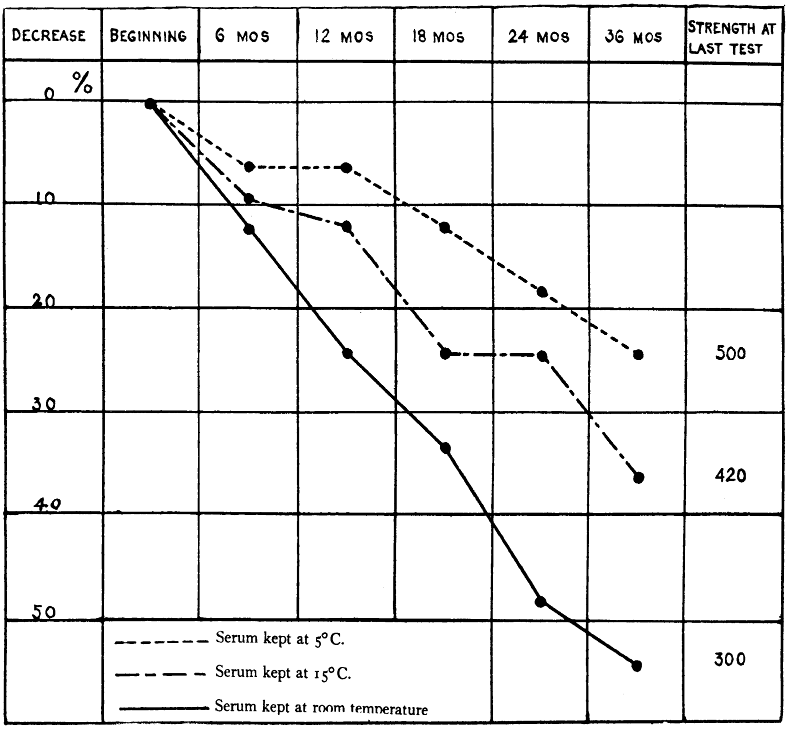

CHART 9.—Serum No. 07635, P.D. Blood drawn May 1, 1905. Serum preserved with 0.4 per cent trikresol. 240 days from collection of serum to beginning of experiment. Strength when experiment was begun, 660 units per c.c.

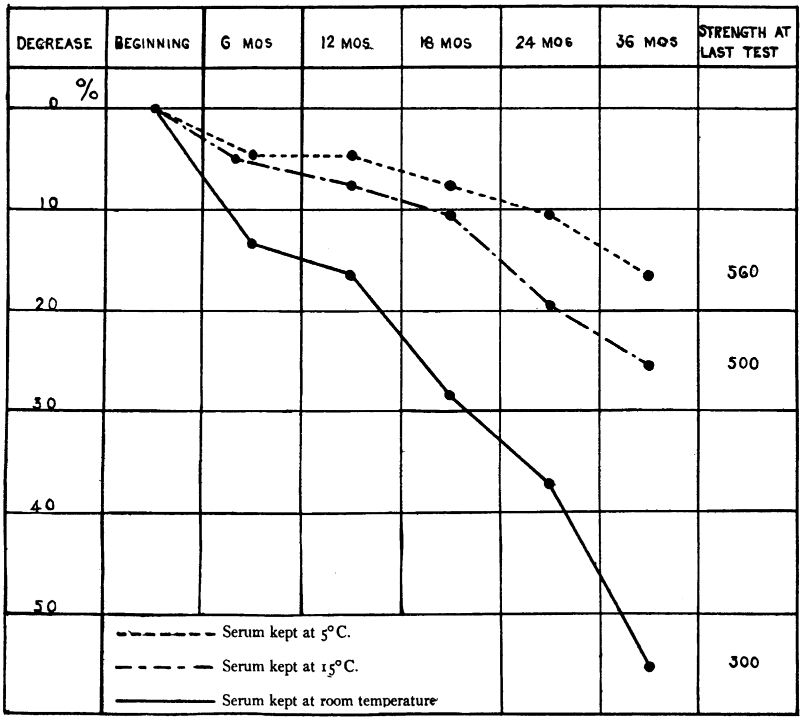

CHART 10.—Serum No. 08022, P.D. Blood drawn September 17, 1905. Serum preserved with 0.4 per cent trikresol. 100 days from collection of serum to beginning of experiment. Strength when experiment was begun, 670 units per c.c.

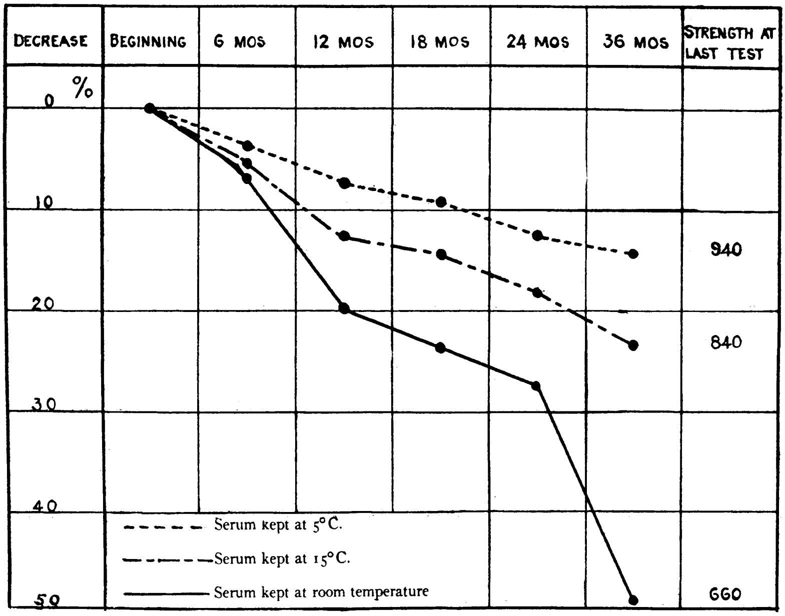

CHART 11.—Serum No. 1,500, F.S.Co. Blood drawn August 21, 1905. Serum preserved with 0.4 per cent trikresol. 374 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,100 units per c.c.

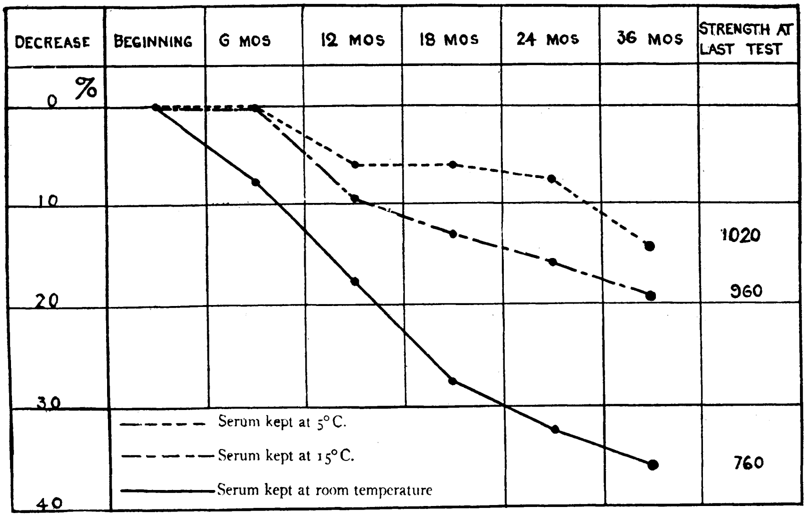

CHART 12.—Serum No. 305, N.Y.H.D. Blood drawn May 18, 1906. Serum preserved with chloroform. 20 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,190 units per c.c.

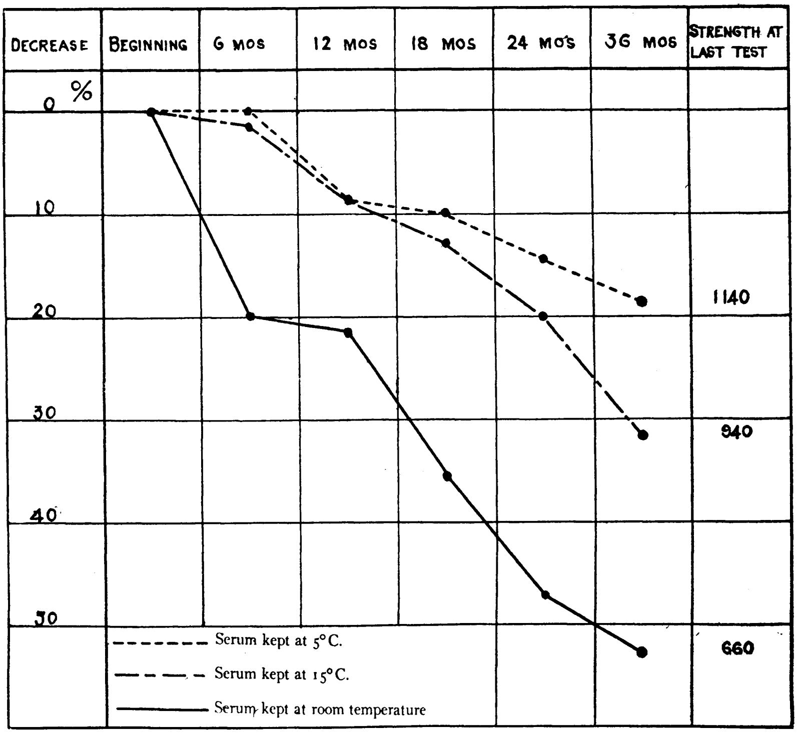

CHART 13.—Serum No. 306, N.Y.H.D. Blood drawn May 18, 1906. Serum preserved with chloroform. 20 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,400 units per c.c.

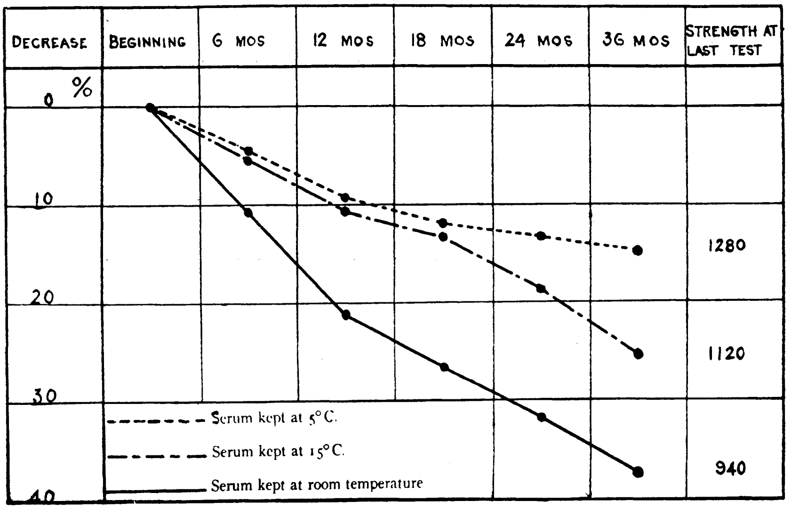

CHART 14.—Serum No. 310, N.Y.H.D. Blood drawn June 4, 1906. Serum preserved with chloroform. 88 days from collection of serum to beginning of experiment. Strength when experiment was begun, 1,500 units per c.c.

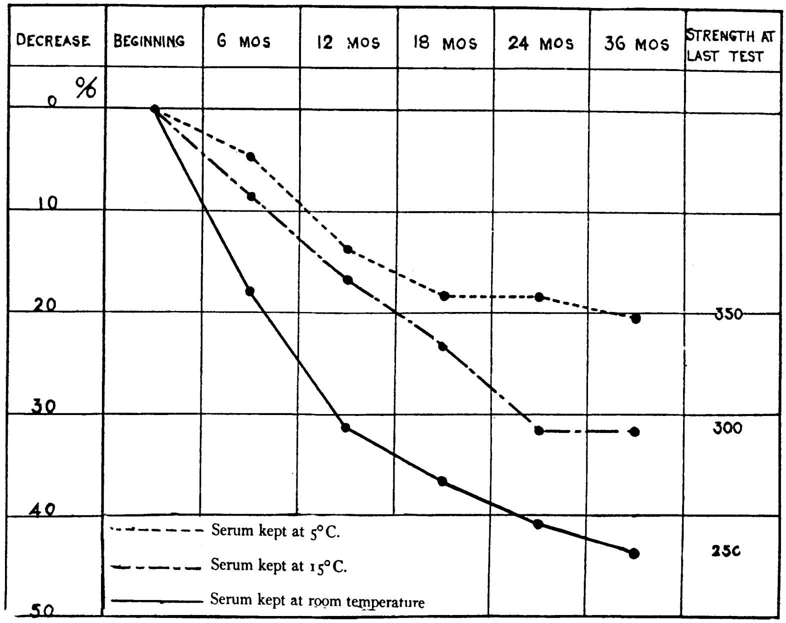

Chart 15.—Serum No. 245, H.M.A. Concentrated after Gibson's method. Concentration completed November 30, 1906. Preserved with chloroform. 35 days from concentration of serum to beginning of experiment. Strength when experiment was begun, 440 units per c.c.

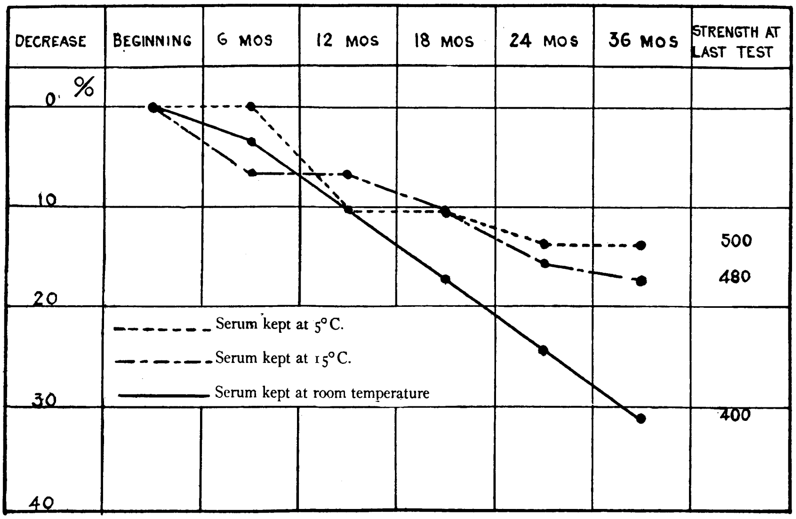

Chart 16.—Serum No. 10B, N.Y.H.D. Concentrated after Gibson's method. Preserved with chloroform. 22 days from concentration of serum to beginning of experiment. Strength when experiment was begun, 580 units per c.c.

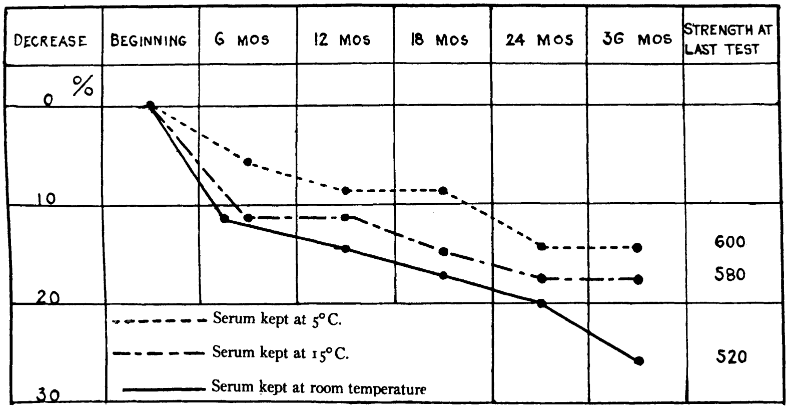

CHART 17.—Serum No. 13C, N.Y.H.D. Concentrated after Gibson's method. Concentration completed February 26, 1909. Preserved with chloroform. 26 days from concentration to beginning of experiment. Strength when experiment was begun, 700 units per c.c.

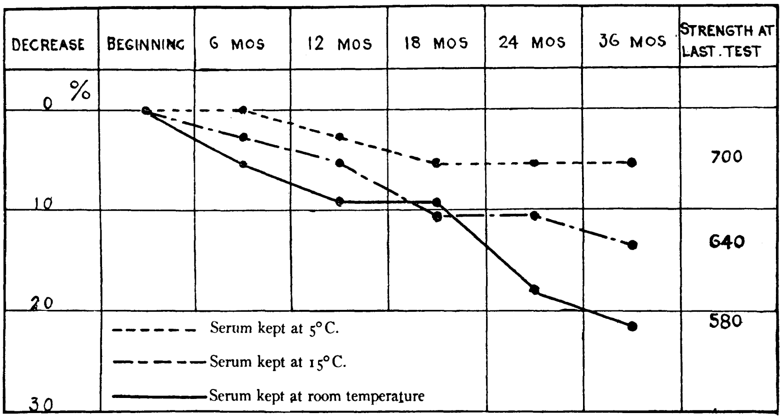

CHART 18.—Serum A249, H.M.A. Concentrated after Gibson's method. Concentration completed December 29, 1906. Preserved with chloroform. 17 days from concentration of serum to beginning of experiment. Strength at beginning of experiment, 740 units per cubic centimeter.

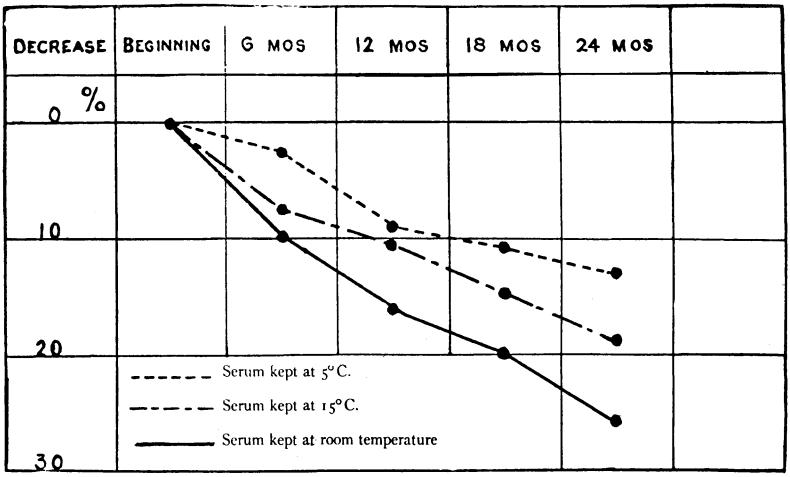

CHART 19.—Showing the average decrease in potency of 14 lots of antitoxic horse serum when kept at 5° C., 15° C., and room temperature, respectively.

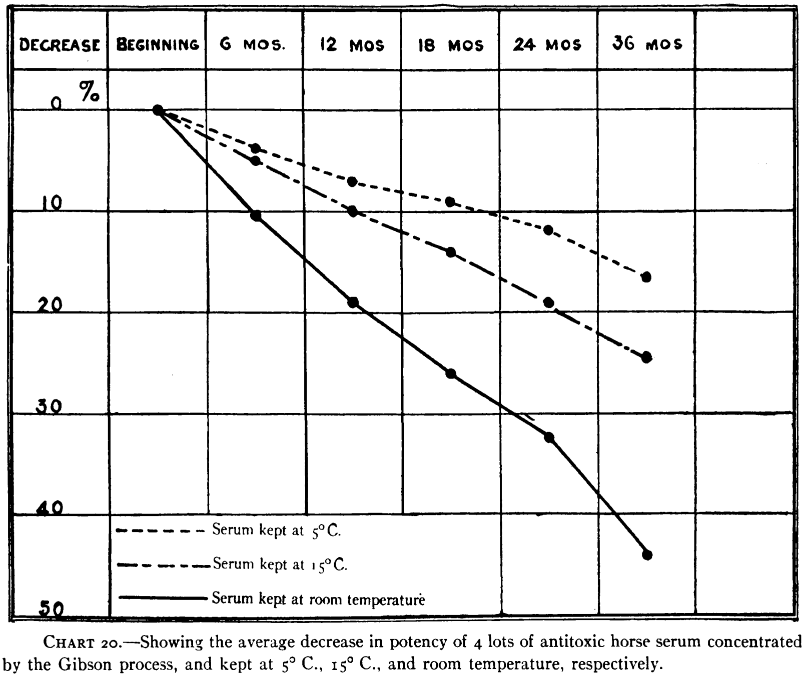

CHART 20.—Showing the average decrease in potency of 4 lots of antitoxic horse serum concentrated by the Gibson process, and kept at 5° C., 15° C., and room temperature, respectively.

as 25 per cent when the serum is kept at room temperature. When kept at 15° C., the average yearly loss is about 10 per cent, altho it may sometimes be double that, but this is exceptional. At 5° C., the average yearly loss falls to about 6 per cent, but there may be instances in which the loss is considerably greater.

The maximum loss of any serum in three years at room temperature was 59 per cent; at 15° C., 36.4 per cent; at 5° C., 24 per cent; the last two being in the same serum.

The minimum loss for three years at room temperature was 34.6 per cent; at 15° C., 16.7 per cent; and at 5° C., 12.5 per cent; the last two being in the same serum.

One of the very interesting points that were brought out by the experiments was as to the relation of the antitoxic content of a serum to its therapeutic value. It was found that in the test mixtures the protective value of the serum was in exact accord with its unit value; for example, 1/1,500 c.c. of Serum 310 was of the same protective value as 1/150 c.c. of Serum 1,110; the first serum contained 1,500 units per c.c. and the last only 150 per c.c. There can be no question as to the protective value of the serum being in direct relation to its unit value for, in our 200 determinations of the antitoxic potency of serum in this study, the same result was had in each instance.

It was noticed that all sera, even of the same age and kept under the same conditions, did not deteriorate in the same ratio; just why this should be so I am unable to decide, but it is apparently independent of external influences and probably depends on some inherent property of the serum.

The addition of preservatives, such as chloroform and trikresol, apparently did not exert any influence upon the deterioration of the antitoxin as there was no appreciable difference in the behavior of the sera preserved with these two substances.

The opinion held by some physicians that old sera or sera on which the return dates have expired should not be used is without scientific basis, and the only reason for not using such sera would be that a larger amount would have to be given to make up for the decrease in potency. Old sera, unit for unit, are just as potent as fresh sera, and would be, perhaps, less apt to cause severe serum reactions than fresh sera.

CONCLUSIONS.

The average yearly loss in potency of diphtheria antitoxin at room temperature is about 20 per cent; at 15° C., about 10 per cent; at 5° C., about 6 per cent, altho in some instances these percentages may be much increased.

As a result of this work, there appears to be but little difference in the keeping qualities of untreated sera and sera concentrated by the Gibson process.

Diphtheria antitoxin to be placed upon the market and there kept under unknown conditions as regards temperature should not be labeled with a return date longer than two years and should contain an excess of at least 33 per cent to allow for decrease in potency; in addition, when the serum is sold in syringes with an absorbable piston, an excess should be added for this loss.

Dried diphtheria antitoxin kept in the dark, at 5° C., retains its potency practically unimpaired for at least 5½ years.

The lack of confidence in the therapeutic properties of old sera is without basis, as such sera, unit for unit, are as potent as new sera.

The protective value of diphtheria antitoxin is in exact accord with its unit value, and is independent of the volume of the serum or other properties in the serum.